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Rapid Communication

A Reliable Method for Simultaneous Demonstration of Two Antigens Using a Novel Combination of Immunogold–Silver Staining and Immunoenzymatic Labeling[1]

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We have developed a reliable and sensitive immunohistochemical staining technique which allows the simultaneous demonstration of two different antigens expressed in or on the same cell (referred to as mixed labeling), together with the evaluation of the general histopathological appearance of the tissue. The staining procedure combines a three-step (streptavidin–biotin) immunogold–silver staining (IGSS) with a three-step immunoenzymatic labeling. For this purpose, we investigated the compatibility of IGSS with various substrates of peroxidase or alkaline phosphatase (AP). Highly reliable and discernible mixed labeling was achieved only after initial labeling with IGSS followed by AP labeling using the substrates naphthol AS-MX phosphate/Fast Blue or naphthol AS-BI phosphate/New Fuchsin, respectively. To ensure utmost specificity, we applied FITC-conjugated mouse monoclonal antibodies and rabbit anti-FITC immunoglobulins visualized by AP-labeled immunoglobulins and the respective substrate in a final step. This novel approach provides an excellent means for demonstration of immunocompetent cells and unequivocal determination of the percentage of specific cell subsets in infiltrated tissue. The advantages of this method, as compared with double immunofluorescence or double immunoenzymatic labeling, were investigated and are discussed. (*J Histochem Cytochem 38:307–313, 1990*)

KEY WORDS: Immunohistochemistry; Immunogold–silver staining; FITC–anti-FITC system; Leucocyte subpopulations; Two-color staining.

Introduction

Double immunoenzymatic labeling (IEL) of cryostat sections has become a widely applied technique in immunohistology since its first description (23). Its major advantage is based on the possibility of studying both labels simultaneously. This is in contrast to double immunofluorescence (IF) procedures (17,30), in which the use of different filters and the invisibility of the general histology during fluorescence microscopy constitute considerable disadvantages. In addition, the fading of color signals does not allow long-term documentation and retrospective evaluation of IF-stained tissue samples. On the other hand, the use of double IEL methods for identification of two different surface or cytoplasmic antigens of the very same cell or of other tissue components (referred to as mixed labeling) is associated with fundamental difficulties that have not yet been overcome (4,19). Thus far, all the enzymatic color reactions that have been combined either interfered with each other or resulted in markedly decreased sensitivity (24). These are the major reasons for the more frequent use of double IF staining procedures despite the disadvantages mentioned above.

In a variety of diseases, analysis of immunocompetent cells infiltrating the affected tissue is important for understanding the pathogenetic mechanisms (25,26,28). In addition to the identification of various lymphocyte populations (B-cells, $CD4^+$ and $CD8^+$ T-cells), the demonstration of lymphocyte subsets expressing memory or activation antigens (interleukin-2 receptor, HLA-DR antigen, CD44, CD45RO, transferrin receptor) is of utmost significance. Our interests focus on the analysis of these subsets in various skin disorders (acute and chronic skin inflammation, autoimmune disorders) to evaluate their role in the course of disease and to gain further insight into the immunopathological mechanisms that become effective. In view of the importance for these analyses of a mixed labeling system, we investigated the possibility of a novel staining protocol combining an IGSS (9,15) with an immunoenzymatic method. We evaluated the various combinations of IGSS and either peroxidase (POX) or alkaline phosphatase (AP) labeling, using different substrates for the enzymatic reactions. Our

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results demonstrate that only the initial staining with IGSS, followed by AP staining with the substrates naphthol AS-MX phosphate (NAMP)/Fast Blue or naphthol AS-BI phosphate (NABP)/New Fuchsin, resulted in excellent mixed labeling. All other combinations tested did not produce satisfactory results. In addition to the three-step IGSS (6,7), the application of FITC-conjugated monoclonal antibodies (MAb) and rabbit anti-FITC immunoglobulins (Ig) (14,31,32) detected by AP-labeled anti-rabbit Ig ensured the high sensitivity of the second label. As a result, immunocompetent cells that are positive for one or for two antigens are clearly discernible. This novel method therefore combines the aforementioned advantages of both double IEL and double IF.

Materials and Methods

Preparation of Skin Sections. Fresh human biopsy specimens of inflamed skin were obtained for routine histology from patients suffering from atopic dermatitis, eczema, and mucosis fungoides. Some of the material was snap-frozen in Tissue-Tek OCT compound (Miles Scientific; Naperville, IL) using isopentane pre-cooled in liquid nitrogen. Cryostat sections (4 μm) were thawed onto slides coated with Histostik (Accurate Chemicals; San Diego, CA), air-dried, and fixed in acetone (4°C, 10 min). Gelatin-coated slides are not recommended for IGSS. To clear slides from OCT compound and to block nonspecific reactions, they were rinsed in a solution (Blotto) containing 5% skim milk powder and 0.1% Tween 20 in PBS, pH 7.4. Blotto was used in all further washing steps unless otherwise stated. For blocking of Fc receptors, sections were pre-incubated for 20–30 min at room temperature (RT) in Blotto containing 20% sheep serum.

Mouse MAb, Conjugated Polyclonal Antibodies, and Conjugates. All MAb applied in the present study are commonly used and all reagents are commercially available. The dilutions and sources of antibodies and conjugates are summarized in Table 1.

IGSS. For IGSS (8), a three-step procedure according to Coggi et al. (7) and Bronckers et al. (6) was used in all experiments. The sections were incubated with the first-step antibodies (for dilution see Table 1) for 10–14 hr (overnight) at 4°C. Thereafter, the slides were thoroughly washed in Blotto three times and the biotin-conjugated second-stage antibodies were allowed to remain on the sections for 1 hr at RT. After a brief wash in Blotto, all following washes were performed in Tris rinsing buffer (0.05 M, pH 7.6) containing Tween 20 (0.1%) and BSA (0.2%). Streptavidin-conjugated gold was overlaid for 1 hr at RT, and after three washes in Tris rinsing buffer followed by three washes in distilled water, freshly prepared silver enhancement mixture Intense SE TM II (Janssen Life Science; Olen, Belgium) was applied for 5–12 min. The silver enhancement process was monitored under the microscope using brightfield or epipolarization illumination (10).

POX/AP IEL. The sections were incubated with FITC-conjugated antibodies (Table 1) overnight at 4°C and were subsequently labeled with rabbit anti-FITC Ig and sheep anti-rabbit Ig conjugated with POX or AP. Between each incubation step (RT, 1 hr), the sections were rinsed with Blotto, and in the final washing step, before addition of substrates, with Tris rinsing buffer. The labeling was visualized with the substrates diaminobenzidine (DAB) (Sigma Chemicals; St Louis, MO) and 3-amino-9-ethylcarbazole (AEC) (Sigma) for the POX reaction, and NAMP/Fast Red (Sigma), NAMP/Fast Blue (Sigma), NABP/New Fuchsin (Sigma; or Merck, Darmstadt, FRG), and bromochloro-indolyl-phosphate (BCIP)/nitroblue tetrazolium (NBT) (Sigma) for the AP reaction. The DAB solution (12,16) contained 0.5 mg/ml DAB, 0.01% (w/v) cobalt chloride, 6.8 mg/ml imidazole (29), and 0.03% (v/v) H_2O_2 in PBS (pH 7.4), and the AEC solution (13) contained 0.2 mg/ml AEC, previously dissolved in *N,N*-dimethylformamide (final concentration 5%), 0.015% H_2O_2 in acetate buffer (50 mM, pH 5). The NAMP/Fast Red and NAMP/Fast Blue solutions contained 0.2 mg/ml NAMP dissolved in *N,N*-dimethylformamide (final concentration 2%), 1 mM levamisole, and 1 mg/ml Fast Red TR salt or Fast Blue TR salt, respectively, in Tris buffer (0.1 M, pH 8.2). The NABP/New Fuchsin solution (22) consisted of 0.5 mg/ml NAPB dissolved in *N,N*-dimethylformamide (final concentration 0.5%), 6 mM sodium nitrite, 1 mM levamisole, and 4% (w/v) New Fuchsin (stock solution diluted in 2 M HCl) in Tris buffer (0.2 M, pH 9.0). The BCIP/NBT solution (20) contained 0.38 M BCIP (50 mg/ml dissolved in *N,N*-dimethylformamide) and 0.4 M NBT (75 mg/ml dissolved in 70% *N,N*-dimethylformamide) in Tris-buffered saline (100 mM, pH 9.5). The substrate solutions were filtered onto the slides and color development was stopped after 2 min in the case of DAB and after 5–20 min when other substrates were used.

Combination of IGSS With POX/AP Labeling. For the mixed labeling with IGSS and IEL, the following sequence of treatments was shown to give the best results: Blotto containing 20% sheep serum (20–30 min, RT); mouse MAb for first (IGSS) labeling (overnight, 4°C); biotinylated

Table 1. *Antibodies and Conjugates*

Antibodies/complexes	Dilution	Source
Anti-HLA-DR	1/30–1/100	Becton–Dickinson (Sunnyvale, CA)
Anti-HLA-DR, FITC-conjugated	1/60–1/200	Becton–Dickinson
Anti-CD45RO (UCHL-1)	1/30–1/60	Dakopatts (Copenhagen, Denmark)
Anti-CD45RO (UCHL-1), FITC-conjugated	1/60–1/100	Dakopatts
Anti-CD45 (HLe)	1/30–1/100	Becton–Dickinson
Anti-CD45 (HLe), FITC-conjugated	1/60–1/100	Becton–Dickinson
Anti-Leu-4 (CD3), FITC-conjugated	1/30–1/100	Becton–Dickinson
Anti-Leu-2a (CD8), FITC-conjugated	1/30–1/100	Becton–Dickinson
Anti-mouse Ig, biotin-conjugated	1/100–1/200	Amersham (Amersham, UK)
Rabbit anti-FITC Ig	1/500–1/1000	East Acres Chemicals (Southbridge, MA)
Goat anti-rabbit Ig, POX-conjugated	1/200	Accurate Chemicals (San Diego, CA)
Goat anti-rabbit Ig, AP-conjugated	1/200	Accurate Chemicals
Donkey anti-rabbit Ig, FITC-conjugated	1/200	Jackson Immuno Research (Avondale, PA)
Streptavidin-conjugated gold	1/40–1/50	Janssen (Olen, Belgium)
Streptavidin-conjugated Texas Red	1/50	Amersham

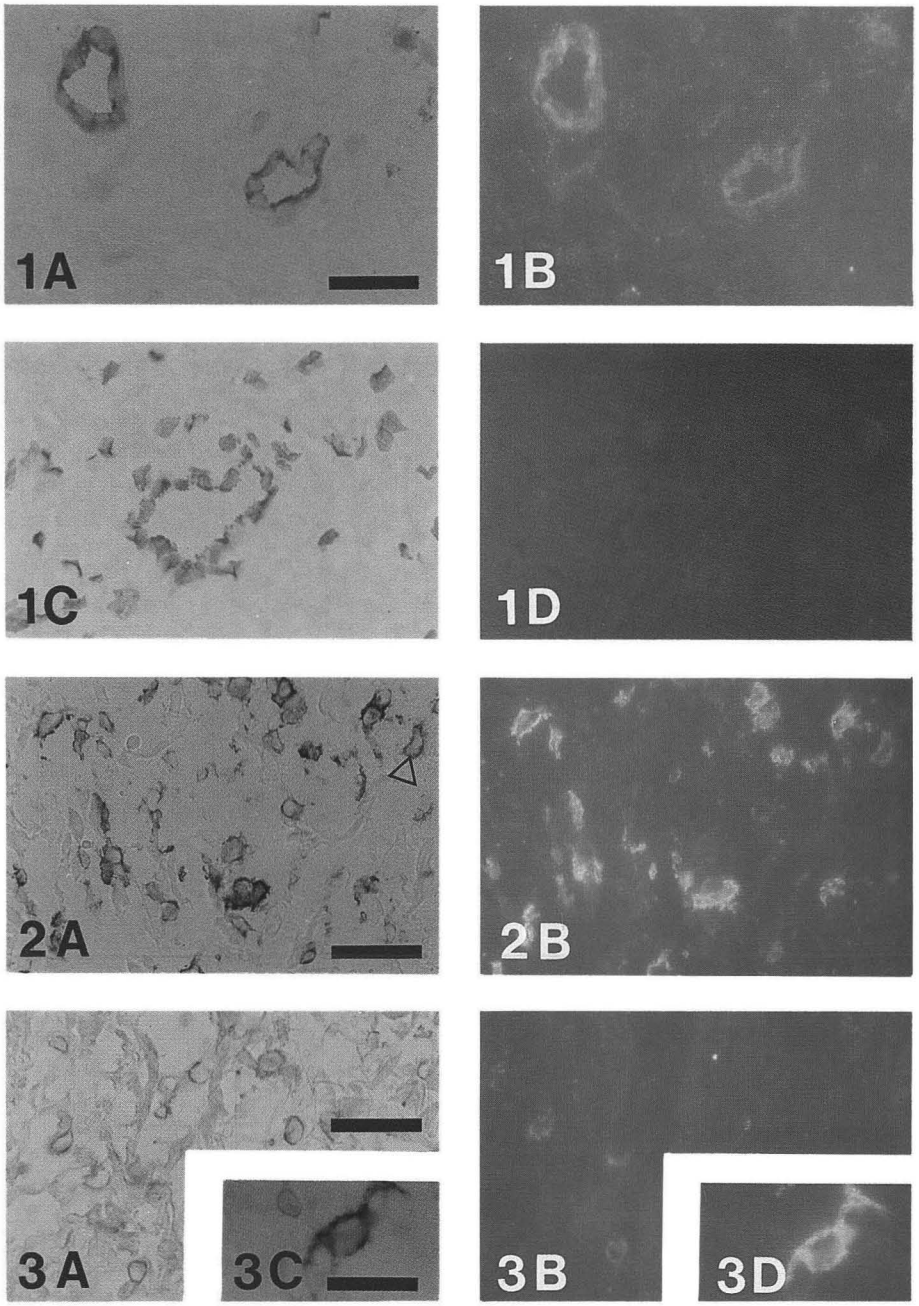

Figure 1. (**A**) Dermal vessels stained for HLA-DR antigen expression by a three-step IEL with a final AP-conjugated Ab and NAMP/Fast Red as substrate; hematoxylin counterstaining. (**B**) The same area as in **A** under epipolarization illumination shows an intrinsic shining of the NAMP/Fast Red color product similar to IGSS. (**C**) Dermal vessel stained for HLA-DR antigen as in **A**, but with NABP/New Fuchsin as substrate; hematoxylin counterstaining. (**D**) Same area as in **C** under epipolarization illumination shows no intrinsic incident polarized light reflection. Bar = 25 μm.

Figure 2. (**A**) Skin section with mycosis fungoides stained for CD3 with IGSS and for HLA-DR with AP–NAMP/Fast Blue. The figure shows mutually exclusive expression of the two antigens, with only a few cells being doubly positive (arrowhead). Brightfield illumination. (**B**) NAMP/Fast Blue labeling does not show incident polarized light reflection and therefore does not interfere with IGSS. Bar = 25 μm.

Figure 3. Double staining with IGSS (CD8) and AP–NAPB/New Fuchsin (HLA-DR) in a skin lesion of atopic dermatitis (**A**) under brightfield illumination. (**B**) Only a few cells co-express both antigens and are therefore IGSS positive (epipolarization illumination). (**C**) The same tissue stained with AP–NAMP/New Fuchsin (CD45) and IGSS (HLA-DR) under brightfield and (**D**) under epipolarization illumination. Bars: **A** = 25 μm; **C** = 16 μm.

sheep anti-mouse Ig antibodies (1 hr, RT); streptavidin-conjugated gold (1 hr, RT); blocking with 10% mouse serum in Blotto (20 min, RT); FITC-conjugated MAb for the second labeling (IEL) (overnight, 4°C or 2 hr at RT); rabbit anti-FITC Ig (1 hr, RT); POX/AP-conjugated goat anti-rabbit Ig (1 hr, RT), IGSS enhancement mixture (5–12 min, RT); POX/AP substrate (2–20 min). The IGSS enhancement, as well as the AP-substrate reaction, should be monitored from time to time under a microscope.

Double IF. For double IF, streptavidin-conjugated Texas Red was used instead of streptavidin-conjugated gold (see Table 1), and FITC-conjugated donkey anti-rabbit Ig antibodies (Table 1) instead of enzyme-conjugated antibodies.

Counterstaining and Microscopic Analysis of Sections. The sections were counterstained with hematoxylin, where indicated and mounted (Gurr Aquamount; BDH, Poole, UK). For evaluation a Zeiss Axiophot microscope equipped for polarized incident light (epipolarization) microscopy (Zeiss; Oberkochen, FRG) was used.

Results

Single IGSS and Single IEL

For one-color staining with either IGSS or IEL, our results confirmed

previous reports on these methods (6,7,10,15). The three-step procedure used for IEL was based on an FITC–anti-FITC system (14,31,32) employing the fluorochrome as a hapten, and showed very specific staining regardless of the fluorochrome/protein ratio of the fluoresceinated MAb. The use of Blotto as rinsing buffer (manuscript in preparation) was particularly beneficial for the signal-to-noise ratio, because it significantly reduced the background staining for both labeling procedures. For all the primary MAb used we found a significantly higher sensitivitiy for IEL as compared with IGSS. For IEL, the MAb could be used in a three- to fivefold higher dilution. Epipolarization microscopy, although lowering the detection threshold of gold-labeled antigens for IGSS, could not fully compensate for the high sensitivity of the three-step IEL, which is comparable to three-step streptavidin–biotin POX/AP methods in terms of sensitivity.

IGSS Combined With IEL

When, after establishing the conditions for IGSS and IEL, both staining protocols were combined for double labeling, the sequence of labeling was found to be critical for specificity and sensitivity. Performance of IEL before IGSS produced nonspecific IGSS signals of cells already labeled with the substrates of POX or AP (results not shown). This can be partly explained by silver precipitation on the color substrates of the preceding IEL. However, a highly specific color enzyme reaction was obtained using the IGSS procedure before the more sensitive IEL. The latter yields specific and stronger signals of the second antigen to be detected. For these reasons, IGSS without silver enhancement was carried out first in all further double mixed labeling experiments, followed by IEL, whereby the silver enhancement was performed before the AP/POX–substrate reaction. The combination of IGSS with POX labeling, using DAB as substrate, resulted in double staining with poorly discernible color products. In contrast, the POX substrate AEC yields a red reaction product and therefore appeared more suitable to be combined with IGSS. However, the POX reaction with AEC almost completely neutralized the IGSS and resulted in insufficient staining. In summary, the two most widely used POX substrates are not compatible with IGSS for double mixed labeling.

For this purpose, in the next series of experiments, an AP staining protocol for the second antigenic determinant was established which would be suitable for combination with IGSS. The four most commonly used substrates, NAMP/Fast Red, NAMP/Fast Blue, NABP/New Fuchsin, and BCIP/NBT, were applied. All substrates perfectly visualized the antigenic determinant (e.g., HLA-DR). As could be expected, the dark purple color product of BCIP/NBT was difficult to discriminate from IGSS under light microscopic visualization and therefore is not an appropriate substrate for mixed labeling. NAMP/Fast Red was not suitable for this purpose either. Under brightfield illumination, its red reaction product was clearly visible (Figure 1A) and could be expected to contrast well with the brown color of IGSS; however, the NAMP/Fast Red reaction product reflected polarized incident light (Figure 1B) in a way similar to IGSS. Under epipolarization illumination, only NABP/New Fuchsin (Figure 1D) and NAMP/Fast Blue (Figure 2B) did not exhibit any intrinsic polarized light reflection and nevertheless contrasted well with IGSS under brightfield illumination (Figures 2, 3, and 4).

Therefore, various combinations of MAb to be utilized for mixed labeling were examined using NAMP/Fast Blue and NABP/New Fuchsin as substrates for AP. Both substrates performed equally well in terms of sensitivity, but the preparation of NAMP/Fast Blue is simpler and less time consuming. To find out whether this mixed labeling procedure is specific and sensitive enough to detect antigens that are co-expressed on immunocompetent cells, we applied the corresponding MAb in two reciprocal combinations ("reversed order incubation"). For example, HLA-DR expression was traced with IGSS and CD45 expression with AP, as well as vice versa. Subsequently, all the singly and doubly positive cells were counted in both sections. We found an identical distribution of single- and double-labeled cells (data not shown). This result clearly demonstrated the high specificity of the presented mixed labeling method. By this technique, singly and doubly positive cells could even be distinguished under epipolarization illumination. IGSS single-labeled cells displayed yellow bright grains, whereas cells additionally labeled with AP (NAMP/Fast Blue) showed a shift of the bright yellow color towards a bright blueish color (Figures 4C, 4D, 5A, and 5B). This beneficial effect was primarily seen with NAMP/Fast Blue, which was therefore used preferentially.

In summary, the mixed labeling is a powerful tool to define singly and doubly positive cells in frozen sections. Using this technique, we can analyze the distribution of activated T-lymphocytes in inflammatory lesions by staining for CD3 or CD8 and CD45RO expression (Figures 5A, and 5B). $CD3^+$ cells are visualized by IGSS, and the relatively high proportion of activated cells are co-expressing CD45RO, which is unambiguously seen by the additional blue AP label.

The sensitivity of mixed labeling was compared to double IF, for which AP and gold conjugates were replaced by FITC and Texas Red conjugates, respectively (data not shown). As judged by the dilution of the primary Ab, the sensitivity of IF was similar or up to two times higher for tracing the first antigen. For detection of the second antigen, however, the sensitivity of mixed labeling was two to three times higher.

Discussion

In the present study we demonstrated a novel combination of IGSS with IEL which allows simultaneous and unambiguous demonstration of antigens co-expressed on the same cell. This technique is referred to as mixed labeling. The staining protocol developed allows highly specific and sensitive double labeling, provided that IGSS is performed before IEL. Among many AP-specific substrates tested, only NAMP/Fast Blue and NABP/New Fuchsin revealed signals that did not interfere with IGSS under either brightfield or epipolarization illumination. The use of FITC-conjugated primary MAb for IEL (31,32) prevents any crossreactivity with the antigen detected by IGSS and supports the specificity of the procedure. The designed method does not require conjugation of primary MAb with haptens such as arsanilic acid (11), which bears the inherent risk of loss of antibody activity or inefficient coupling. The applicability and practicability is facilitated by the utilization of commonly and commerically available substances. For this reason, it can easily be applied for routine work and diagnostic purposes.

As compared with double IF techniques (24), this new method

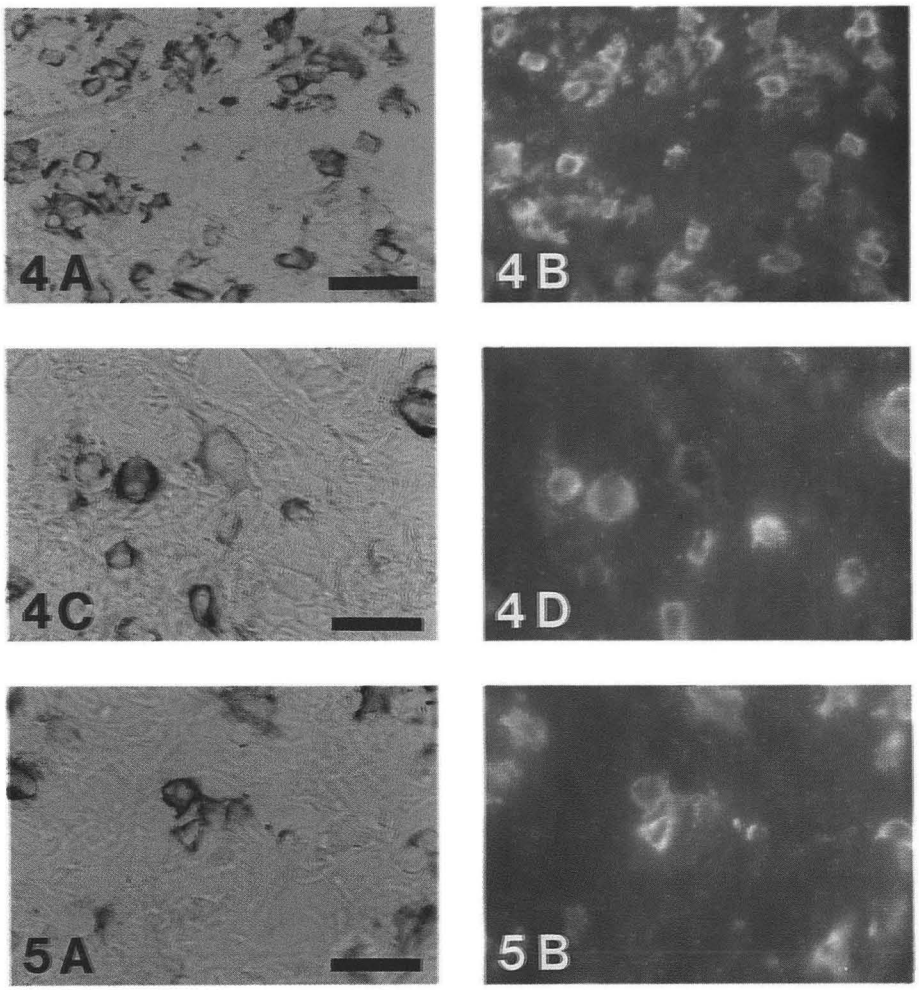

Figure 4. Double staining for CD45 (IGSS) and HLA-DR (AP–NAMP/Fast Blue) in a skin section of mycosis fungoides. (**A**) A high percentage of cells are doubly positive for CD45 and HLA-DR under brightfield illumination and (**B**) under epipolarization illumination. (**C, D**) The same section with higher magnification. Single IGSS-positive cells show yellow grains, whereas double-labeled cells are light blue. Bars: **A** = 25 μm; **C** = 16 μm.

Figure 5. Double staining for CD3 (IGSS) and CD45RO (AP–NAMP/Fast Blue). Detail with two $CD3^+$ lymphocytes. One lymphocyte is activated ($CD45RO^+$), demonstrated by the additional blue label (**A**), which appears light blue under epipolarization illumination (**B**). Bar = 16 μm.

offers several important advantages. First, both color products of the mixed labeling are visible under the same lighting (brightfield), although switching to epipolarization illumination increases and clarifies the IGSS signal. Second, this method enables the discrimination of cell and tissue morphology with concomitant use of routine histological stains. Therefore, pathological irregularities and notable details can easily be revealed. Third, as opposed to IF, IGSS and IEL do not fade and therefore allow long-term documentation and re-examination of the section at a later date.

For detection of the second antigen, mixed labeling using FITC-conjugated MAb was two to three times more sensitive than a similar three-step IF procedure. For staining of the first Ag, however, we found a slightly lower sensitivity of IGSS as compared with IF. This has been encountered in several approaches (1), whereas others revealed a fairly high sensitivity for IGSS, especially when intracellular antigens had been labeled (6,15). The difference in sensitivity of IGSS and IEL should therefore be taken into account when a mixed double-labeling protocol is being used. For this reason, it is advisable to stain the less abundant antigen by IEL.

The mixed labeling achieved with brown IGSS and well-contrasting blue or red AP labeling is clearly visible and of better quality than mixed IEL. The latter is difficult to evaluate, especially in the case of a low-density second antigen that does not allow strong labeling. As a result, unequivocal distinction from single-stained cells is hardly possible. In addition, it is difficult to find an appropriate substrate and enzyme combination for double IEL. In our hands, the combination of blue and red dyes often resulted in continuous change of hue, making assessment unfeasible. In general, mixed labeling with double IEL has not gained much application and has been described as not reliable by several authors (4,17,19). The problems mentioned above can be avoided using the combination of IGSS and only one appropriate IEL. As described in the present study, the enzyme AP, rather than POX, appears to be most suitable for mixed labeling with IEL and IGSS, because it does not interfere with any endogenous POX activity. The basic chemophysical differences between both labels offer the advantage of avoiding mutual interference. This would also apply for the combination of autoradiography and IEL (2), but in contrast to IGSS it is more laborious, time consuming, and hazardous. The application of glucoxidase (21) does not seem appropriate for mixed labeling

either, because its diffuse color localization may interfere with the label of adjacent cells which, as a result, may be mistaken as double labeled.

The 5-nm gold particles that have previously been shown to be superior for IGSS (10) and which, unlike larger particles (e.g., 20 nm), do not exhibit steric hindrance, are visible under epipolarization illumination. This facilitates the detection of double-labeled cells and even allows the semiquantitative assessment of antigen density. We could detect $CD45^+$ cells (Fast $Blue^+$) exhibiting different levels of HLA-DR antigen expression, as demonstrated by a low or high density of shining grains. The contrast between IGSS (under epipolarization illumination) and AP (brightfield illumination) can be used for computerized automated image analysis. In addition, both mixed labeling and double IF allow documentation with black-and-white photographs, whereas double IEL requires colored documentation.

It may be beneficial, for time reduction, to adapt the mixed labeling for use with a novel capillary action staining system recently developed by Brigati et al. (5). Its advantages have already been demonstrated by Kumar (18) for single IGSS. Additional saving of time can also be achieved by simultaneous incubation of two reagents (e.g., streptavidin–gold and FITC–conjugated Ab).

The present technique, developed to investigate the co-expression of antigens on immunocompetent hemopoietic cells, may very well be beneficially adapted to various other areas of research, such as the demonstration of overlapping hormone production in neuroendocrine cells or the simultaneous detection of viral or bacterial antigens in infected cells. This would also apply for two-color DNA/RNA in situ hybridization with non-radioactive probes or the simultaneous in situ detection of RNA/DNA and antigens (3,27). The present communication adds a further tool to supply the ongoing need for reliable and sensitive immunohistological techniques suitable for analysis of shared epitopes.

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